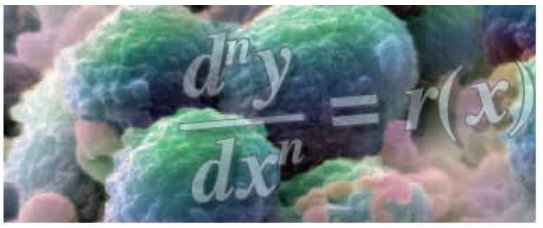

PBCF

SOP

Thawing, Propagating and Cryopreserving Protocol

for

NCI-PBCF-CRL1619

A-375

Malignant melanoma cells

September 12, 2013; Version 1.0

PS-OC Network Bioresource Core Facility

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Protocol for Thawing, Propagating and Cryopreserving
NCI-PBCF-CRL1619 (A-375), (ATCC® CRL-1619™)
Malignant melanoma cells

1. Background Information on A-375 cell line

Designation:	A-375	
Biosafety Level: 1	Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication on the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health.	
Shipping condition:	Frozen (in dry ice)	
Organism:	*Homo sapiens*, human	
Gene Expression:	NRAS mutation	
Growth profile:	See *Appendix 2*	
Karyotyping:	See *Appendix 3*	
Cell Type:	Mesenchyme	
Morphology:	Epithelial-like (see *Appendix 1*)	
Source :	***Organ***	Skin
Source :	***Disease***	Malignant melanoma

For more information visit the ATCC webpage: https://www.atcc.org/Products/All/CRL-1619.asp

2. General Information for the thawing, propagating and cryopreserving of NCI-PBCF- CRL1619 (A-375)

Culture Initiation	• The cryoprotectant (DMSO) should be removed by centrifugation. • The seeding density to use with a vial of A-375 cells is about 1.5 x 10^5 viable cells/cm^2 or one vial into a T-12.5 flask containing 6 mL complete growth medium (DMEM + 10% (v/v) FBS).
Complete growth medium	• The complete growth medium used to expand A-375 cells is DMEM + 10% (v/v) FBS. • The complete growth medium (DMEM + 10% (v/v) FBS) should be pre-warmed before use by placing into a water bath set at 35 °C to 37 °C for 15 min to 30 min. • After 30 min, the complete growth medium (DMEM + 10% (v/v) FBS) should be stored at 2 °C to 8 °C when not in use.
Cell Growth	• The growth temperature for A-375 cells is 37 °C ± 1 °C. • 95 % air atmosphere + 5 CO_2 is recommended.
Growth Properties	• Population doubling time (PDT) is approximately 32h (see *Appendix 2*).
Special Growth Requirements	• Subculture A-375 cells at 85 % to 90 % confluence or when cell density reaches an average of 5.0 x 10^5 viable cells/cm^2.
Cytogenetic Analysis	• See *Appendix 3*
Subculture Medium	• The attached A-375 cells are subcultured using 0.25 % (w/v) trypsin-0.53 mM EDTA (ATCC cat. no. 30-2101). • Subculturing reagents should be pre-warmed before use by placing into a water bath set at 35 °C to 37 °C for 15 min to 30 min. • After 30 min, the subculturing medium should be removed to room temperature until used. Subculturing reagents should be stored at 2 °C to 8 °C when not in use.
Subculture Method	• The attached A-375 cells are subcultured using 0.25 % (w/v) trypsin-0.53 mM EDTA (ATCC cat. no. 30-2101). • The enzymatic action of the trypsin-EDTA is stopped by adding complete growth medium to the detached cells. • A split ratio of about 1:5 with a seeding density of 1 x 105 viable cells/cm2 is used when subculturing the A-375 cells.
Viable Cells/mL/Cryovial	• The target number of viable cells/mL/cryo vial is 2 x 10^6 (acceptable range: 1.5 x 10^6 viable cells/mL to 3 x 10^6 viable cells/mL.
Cryopreservation Medium	• The cryopreservation medium for A-375 is the complete growth medium (DMEM + 10 % (v/v) FBS) containing 5 % (v/v) DMSO) (ATCC cat. no. 4-X).

3. General Procedure to be applied throughout the SOP

Aseptic Technique	• Use of good aseptic technique is critical. Any materials that are contaminated, as well as any materials with which they may have come into contact, must be disposed of immediately.
Traceability of material/reagents	• Record the manufacturer, catalog number, lot number, date received, date expired and any other pertinent information for all materials and reagents used. Record information in the Reagent Lot Traceability Table 4 (*Appendix 6*).
Expansion of cell line	• Record the subculture and growth expansion activities, such as passage number, % confluence, % viability, cell morphology (see Figures 1-3, (*Appendix 1*) and population doubling levels (PDLs), in the table for Cell Expansion (Table 5, *Appendix 7*). Calculate PDLs using the equation in *Appendix 8*.
Medium volumes	• Medium volumes are based on the flask size as outlined in Table 1.
Glossary of Terms	• Refer to Glossary of Terms used throughout the document (see *Appendix 4*).
Safety Precaution	• Refer to Safety Precautions pertaining to the thawing, propagating and cryopreserving of A-375 cells (See *Appendix 9*).

Table 1: Medium Volumes

Flask Size	Medium Volume Range
12.5 cm^2 (T-12.5)	3 mL to 6 mL
25 cm^2 (T-25)	5 mL to 13 mL
75 cm^2 (T-75)	10 mL to 38 mL
150 cm^2 (T-150)	30 mL to 75 mL
175 cm^2 (T-175)	35 mL to 88 mL
225 cm^2 (T-225)	45 mL to 113 mL

4. Reagents

Follow Product Information Sheet storage and/or thawing instructions. Below is a list of reagents for the propagation, subcultivation and cryopreservation of A-375 cells.

Table 2: Reagents for Expanding, Subculturing and Cryopreserving A-375 Cells

Complete growth medium (DMEM + 10 % FBS) reagents	Subculturing reagents	Cryopreservation medium reagents
DMEM (ATCC cat. no. 30-2002) *Dulbecco's Modified Eagle's Medium (DMEM) modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate.* *NOTE: This reduced level of sodium bicarbonate (NaHCO3, 1.5 g/L) is intended for use in 5% CO2 in air. Additional sodium bicarbonate may be required for use in incubators containing higher percentages of CO2.*	Trypsin-EDTA (0.25 % (w/v) Trypsin/0.53 mM EDTA) (ATCC cat no. 30-2101)	DMEM + 10 % FBS
10 % (v/v) FBS (ATCC cat. no. 30-2020)	Dulbecco's Phosphate Buffered Saline (DPBS); modified without calcium chloride and without magnesium chloride (ATCC cat no. 30-2200)	10 % (v/v) FBS (ATCC cat. no. 30-2020)
		5 % (v/v) Dimethyl Sulfoxide (DMSO) (ATCC cat. no. 4-X)

a. Preparation of complete growth medium

The complete growth medium (DMEM + 10 % FBS) is prepared by aseptically combining:

1. 56 mL FBS (ATCC cat no. 30-2020) to a 500 mL bottle of basal medium – DMEM (ATCC cat no. 30-2002).
2. Mix gently, by swirling.

5. Thawing and Propagating of Cells

Reagents and Material:

- Complete growth medium (DMEM + 10 % (v/v) FBS)
- Water bath
- T12.5 cm^2 polystyrene flask
- 15 mL polypropylene conical centrifuge tubes
- Plastic pipettes (1 mL,10 mL, 25 mL)

Method:

1. Place complete growth medium (DMEM 10 % (v/v) FBS) in a water bath set at 35 ^{o}C to 37 ^{o}C.
2. Label T12.5 flask to be used with the (a) name of cell line, (b) passage number, (c) date, (d) initials of technician.
3. Wearing a full face shield, retrieve a vial of frozen cells from the vapor phase of the liquid nitrogen freezer.
4. Thaw the vial by gentle agitation in a water bath set at 35 ^{o}C to 37 ^{o}C. To reduce the possibility of contamination, keep the O-ring and cap out of the water.

   Note: Thawing should be rapid (approximately 2 min to 3 min, just long enough for most of the ice to melt).
5. Remove vial from the water bath, process immediately.
6. Remove excess water from the vial by wiping with sterile gauze saturated with 70 % ethanol.
7. Transfer the vial to a BSL-2 laminar-flow hood.

a. Propagating cells

Method:

1. Add 9 mL of complete growth medium (DMEM + 10 % (v/v) FBS) to a 15-mL conical centrifuge tube.
2. Again wipe the outer surface of the vial with sterile gauze wetted with 70 % ethanol.
3. Using sterile gauze, carefully remove the cap from the vial.
4. With a 1 mL pipette transfer slowly the completely thawed content of the vial (1 mL cell suspension) to the 15-mL conical centrifuge tube containing 5 mL complete growth medium (DMEM + 10 % (v/v) FBS). Gently resuspend cells by pipetting up and down.
5. Centrifuge at 125 x g, at room temperature, for 8 min to 10 min.
6. Carefully aspirate (discard) the medium, leaving the pellet undisturbed.
7. Using a 10 mL pipette, add 6 mL of complete growth medium (DMEM + 10 % (v/v) FBS).
8. Resuspend pellet by gentle pipetting up and down.
9. Using a 1 mL pipette, remove 1 mL of cell suspension for cell count and viability. Cell counts are performed using either an automated counter (such as Innovatis Cedex System; Beckman-Coulter ViCell system) or a hemocytometer.
10. Record total cell count and viability. When an automated system is used, attach copies of the printed results to the record.
11. Plate cells in pre-labeled T-12.5 cm^2 flask at about 1.5 x 10^5 viable cells/cm^2.
12. Transfer flask to a 37 °C ± 1°C incubator.
13. Observe culture daily to ensure culture is free of contamination, culture has not reached confluence. Monitor, visually, the pH of the medium daily. If the medium goes from red through orange to yellow, change the medium.

    Note: In most cases, cultures at high cell density exhaust the medium faster than those at low cell density as is evident from the change in pH. A drop in pH is usually accompanied by an increase in cell density, which is an indicator to subculture the cells. Cells may stop growing when the pH falls from pH 7 to pH 6 and loose viability between pH 6.5 and pH6.

14. If fluid renewal is needed, aseptically aspirate the complete growth medium from the flask and discard. Add an equivalent volume of fresh complete growth medium to the flask. Alternatively, perform a fluid addition by adding fresh complete growth medium (DMEM + 10 % (v/v) FBS) to the flask without removing the existing medium. Record fluid change or fluid addition on the Cell Line Expansion Table (see Table 5 in *Appendix 7*).
15. If subculturing of cells is needed, continue to 'Subculturing cells'.

Note: Subculture when cells are 85-90 % confluent (see photomicrographs, Figures 1 and 2 in Appendix 1).

b. Subculturing cells

Reagents and Material:

- 0.25 % (w/v) Trypsin-0.53 mM EDTA
- DPBS
- Complete growth medium (DMEM (ATCC cat no. 30-2002) + 10 % (v/v) FBS (ATCC cat. no. 30-2020))
- Plastic pipettes (1 mL, 10 mL, 25 mL)
- T-75 cm^2, T-225 cm^2 polystyrene flasks

Method:

1. Aseptically remove medium from the flask
2. Add appropriate volumes of sterile Ca^{2+}- and Mg^{2+}-free DPBS to the side of the flask opposite the cells so as to avoid dislodging the cells (see Table 3).
3. Rinse the cells with DPBS (using a gently rocking motion) and discard.
4. Add appropriate volume of 0.25 % (w/v) Trypsin-0.53 mM EDTA solution to the flask (see Table 3).
5. Incubate the flask at 37 $^{o}C \pm 1\ ^{o}C$ until the cells round up. Observe cells under an inverted microscope every 5 min. When the flask is tilted, the attached cells should slide down the surface. This usually occurs after 5 min to 10 min of incubation.

   Note: Do not leave trypsin-EDTA on the cells any longer than necessary as clumping may result.
6. Neutralize the trypsin-EDTA/cell suspension by adding an equal volume of complete growth medium (DMEM + 10 % (v/v) FBS) to each flask. Disperse the cells by pipetting, gently, over the surface of the monolayer. Pipette the cell suspension up and down with the tip of the pipette resting on the bottom corner or edge until a single cell suspension is obtained. Care should be taken to avoid the creation of foam.
7. Using a 1 mL pipette, remove 1 mL of cell suspension for total cell count and viability.
8. Record total cell count and viability.
9. Add appropriate volume of fresh complete growth medium (DMEM + 10 % (v/v) FBS) and transfer cell suspension (for volume see Table 1) into new pre-labeled flasks at a seeding density of about 1×10^5 viable cells/cm^2 or a split ratio of 1:5.

Label all flasks with the (a) name of cell line, (b) passage number, (c) date, (d) initials of technician.

Table 3: Volume of Rinse Buffer and Trypsin

Flask Type	Flask Size	DPBS Rinse Buffer	Trypsin-EDTA
T-flask	12.5 cm^2 (T-12.5)	1 mL to 3 mL	1 mL to 2 mL
T-flask	25 cm^2 (T-25)	1 mL to 5 mL	1 mL to 3 mL
T-flask	75 cm^2 (T-75)	4 mL to 15 mL	2 mL to 8 mL
T-flask	150 cm^2 (T-150)	8 mL to 30 mL	4 mL to 15 mL
T-flask	175 cm^2 (T-175)	9 mL to 35 mL	5 mL to 20 mL
T-flask	225 cm^2 (T-225)	10 mL to 45 mL	5 mL to 25 mL

c. Harvesting of cells for cryopreservation

Reagents and Material:

- 0.25 % (w/v) Trypsin-0.53 mM EDTA
- DPBS
- Complete growth medium (DMEM (ATCC cat. no. 30-2002) + 10 % (v/v) FBS (ATCC cat. no. 30-2020))
- 50 mL or 250 mL conical centrifuge tube
- Plastic pipettes (1 mL, 10 mL, 25 mL)
- Sterile DMSO
- 1 mL to 1.8 mL cryovials
- Ice bucket with ice

Method:

1. Label cryovials to include information on the (a) name of cell line, (b) passage number (c) date.
2. Prepare cryopreservation medium by adding DMSO to cold complete growth medium (DMEM + 10 % (v/v) FBS) at a final concentration of 5 % (v/v) DMSO. Place cryopreservation medium on ice until ready to use.
3. Aseptically remove medium from the flask
4. Add appropriate volumes of sterile Ca^{2+}- and Mg^{2+}-free DPBS to the side of the flask so as to avoid dislodging the cells (see Table 3).

5. Rinse the cells with DPBS (using a gently rocking motion) and discard.

6. Add appropriate volume of 0.25 % (w/v) Trypsin-0.53 mM EDTA solution to the flask (see Table 3).

7. Incubate the flask 37 ^{o}C ± 1 ^{o}C until the cells round up. Observe cells under an inverted microscope every 5 min. When the flask is tilted, the attached cells should slide down the surface. This usually occurs after 5 min to 10 min of incubation.

   Note: Do not leave trypsin-EDTA on the cells any longer than necessary as clumping may result.

8. Neutralize the trypsin-EDTA /cell suspension by adding an equal volume of complete growth medium (DMEM + 10 % (v/v) FBS) to each flask. Disperse the cells by pipetting, gently, over the surface of the monolayer. Pipette the cell suspension up and down with the tip of the pipette resting on the bottom corner or edge until a single cell suspension is obtained. Care should be taken to avoid the creation of foam.

9. Using a 1 mL pipette, remove 1 mL of cell suspension for total cell count and viability.

10. Record total cell count and viability.

11. Spin cells at approximately 125 xg for 5 min to 10 min at room temperature. Carefully aspirate and discard the medium, leaving the pellet undisturbed

12. Calculate volume of cryopreservation medium based on the count performed at step 9 and resuspend pellet in cold cryopreservation medium at a viable cell density of 2.0 x 10^6 cells/mL (acceptable range: 1.5 x 10^6 to 3 x 10^6) by gentle pipetting up and down.

13. Dispense 1 mL of cell suspension, using a 5 mL or 10 mL pipette, into each 1.0 mL cryovial.

14. Place filled cryovials at 2 ^{o}C to 8 ^{o}C until ready to cryopreserve. A minimum equilibration time of 10 min but no longer than 45 min is necessary to allow DMSO to penetrate the cells.

    Note: DMSO is toxic to the cells. Long exposure in DMSO may affect viability.

6. Cryopreserving cells

Material:

- Liquid nitrogen freezer
- Liquid nitrogen
- Cryomed Programmable freezer (Forma Scientific, cat. no. 1010) **or**
- Mr. Frosty (Nalgene, cat. no. 5100)
- Isopropanol
- Cryovial rack

a. Cryopreservation of cells using a rate-controlled programmable freezer

Method:

A slow and reproducible cooling rate is very important to ensure good recovery of cultures. A decrease of 1 °C per min to -80 °C followed by rapid freeze at about 15 °C to 30 °C per min drop to -150 °C will usually work for most animal cell cultures. The best way to control the cooling process is to use a programmable, rate-controlled, electronic freezer unit. Refer to the manufacturer's handbook for detailed procedure.

i. Using the Cryomed

Starting the Cryopreservation Process

1. Check that the liquid nitrogen valve that supplies the Cryomed is open.
2. Check the gauge to ensure that there is enough liquid nitrogen in the open tank to complete the freeze.
3. Install the thermocouple probe so that the tip is immersed midway into the control fluid

   Note: Be sure that the thermocouple is centered in the vial and the vial is placed centered in the rack. The probe should be changed after three uses or if it turns yellow to ensure accurate readings by the controller during the freezing process. Old medium may have different freezing characteristics.
4. Close and latch Cryomed door.
5. Turn on microcomputer, computer and monitor.
6. Double click the "Cryomed" icon. The machine may need to be pre-programmed for specific cell type and medium.
7. From the top of the screen, select MENU → RUN FUNCTIONS → START RUN.
8. Fill out the box which appears on the screen. Cell line ID; TYPE OF SAMPLE; MEDIA; NUMBER OF SAMPLES.
9. Hit the ESCAPE key and the Cryomed will cool to 4 °C.

10. Once Cryomed chamber has cooled to 4 °C, load cryovials onto racks and close the door.

11. When the Cryomed's chamber temperature and the sample temperature have reached approximately 4 °C; press the space bar to initiate the rate controlled cryopreservation process.

Completing the Cryopreservation Process

12. When samples have reached -80°C, an alarm will sound. To silence this, select ALARM from the options at the top of the screen.
13. Select MENU →RUN FUNCTIONS→ STOP. Hit the ESCAPE key to return to the main menu and select EXIT.
14. Immediately transfer vials to liquid nitrogen freezer.
15. Shut down the microcomputer and then turn off the monitor.

b. Cryopreservation of cells using "Mr. Frosty"

1. One day before freezing cells, add 250 mL isopropanol to the bottom of the container and place at 2 ^{o}C to 8 ^{o}C.
2. On the day of the freeze, prepare cells for cryopreservation as described above.
3. Insert cryovials with the cell suspension in appropriate slots in the container.
4. Transfer the container to a -70 °C to -90 °C freezer and store overnight.
5. Next day, transfer cryovials to the vapor phase of liquid nitrogen freezer.

Note: Each container has 18 slots which can accommodate 18 cryovials in one freeze.

Important information when using the rate-controlled programmable freezer or a manual method (Mr. Frosty) for cryopreservation of mammalian cells.

- Regardless which cooling method is used, it is important that the transfer to the final storage location (between -130 °C and -196 °C) be done quickly and efficiently. If the transfer cannot be done immediately, the vials can be placed on dry ice for a short time. This will avoid damage to cultures by inadvertent temporary warming during the transfer process. Warming during this transfer process is a major cause of variation in culture viability upon thawing.

- Always keep the storage temperature below -130 °C for optimum survival. Cells may survive storage at higher temperatures but viability will usually decrease over time. The

ideal storage container is a liquid nitrogen freezer where the cultures are stored in the vapor phase above the liquid nitrogen.

Note: ATCC does not have experience in the cryopreservation of the A-375 cells by any other method than the Cryomed programmable freezer.

7. Storage

- Store cryopreserved cells in the vapor phase of liquid nitrogen freezer (below -130 °C) for optimum long-term survival.

Note: Experiments on long-term storage of animal cell lines at different temperature levels indicate that a -70 °C storage temperature is not adequate except for very short period of time. A -90 °C storage may be adequate for longer periods depending upon the cell line preserved. The efficiency of recovery, however, is not as great as when the cells are stored in vapor phase of the liquid nitrogen freezer.

APPENDIX 1: PHOTOMICROGRAPHS OF NCI-PBCF-CRL1619 (A-375) CELLS

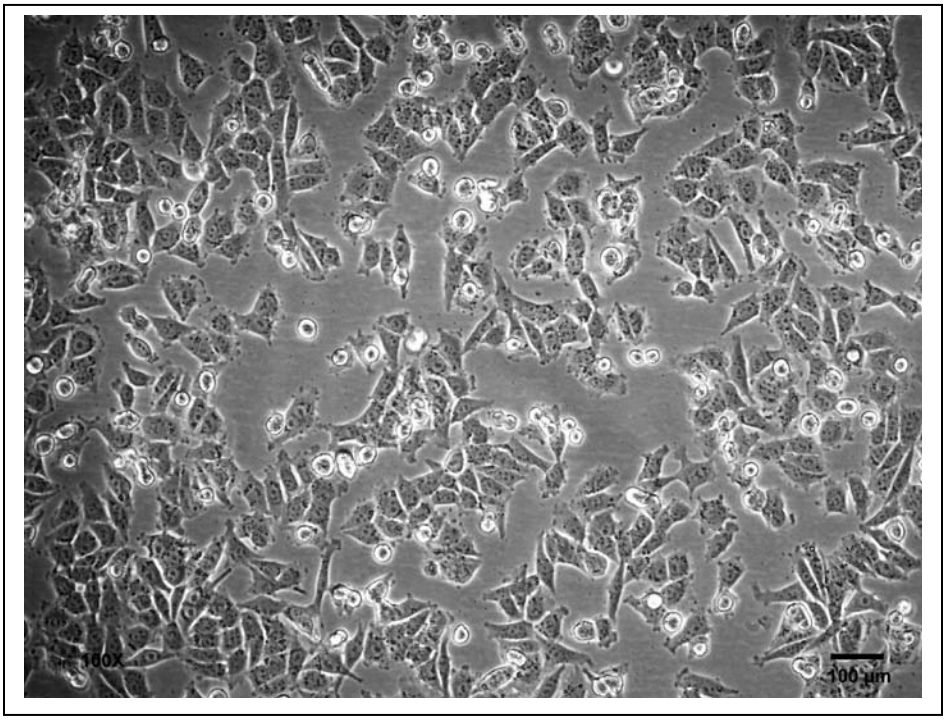


Figure 1: Photomicrograph of A-375 cells after one day, post-subculture. Cells were plated at 1.5 x 10^5 viable cells/cm^2.

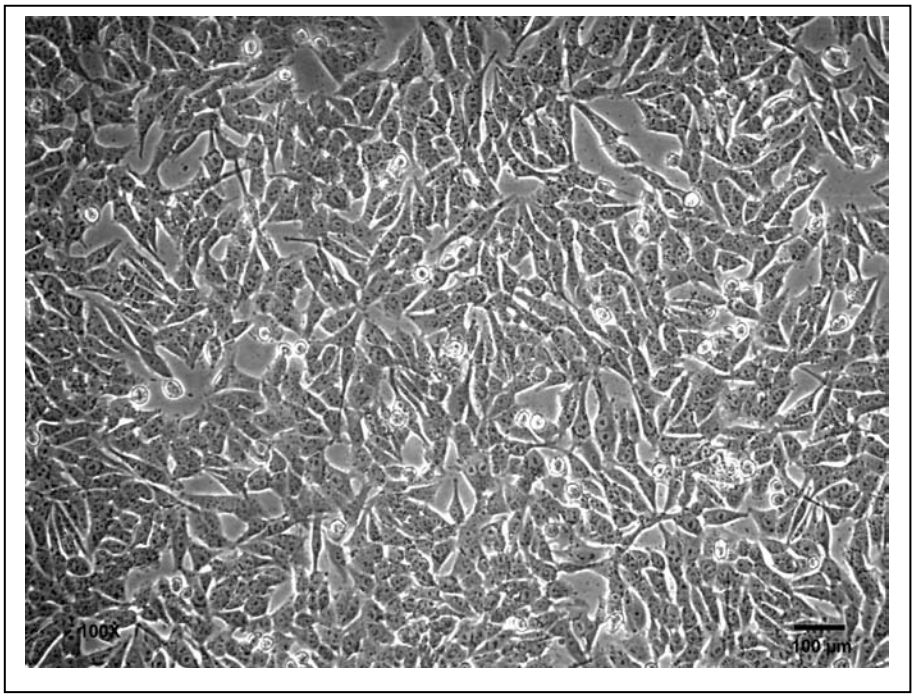


Figure 2: Photomicrograph of A-375 cells after two days, post-subculture. Cells were plated at 1.5 x 10^5 cells/cm^2.

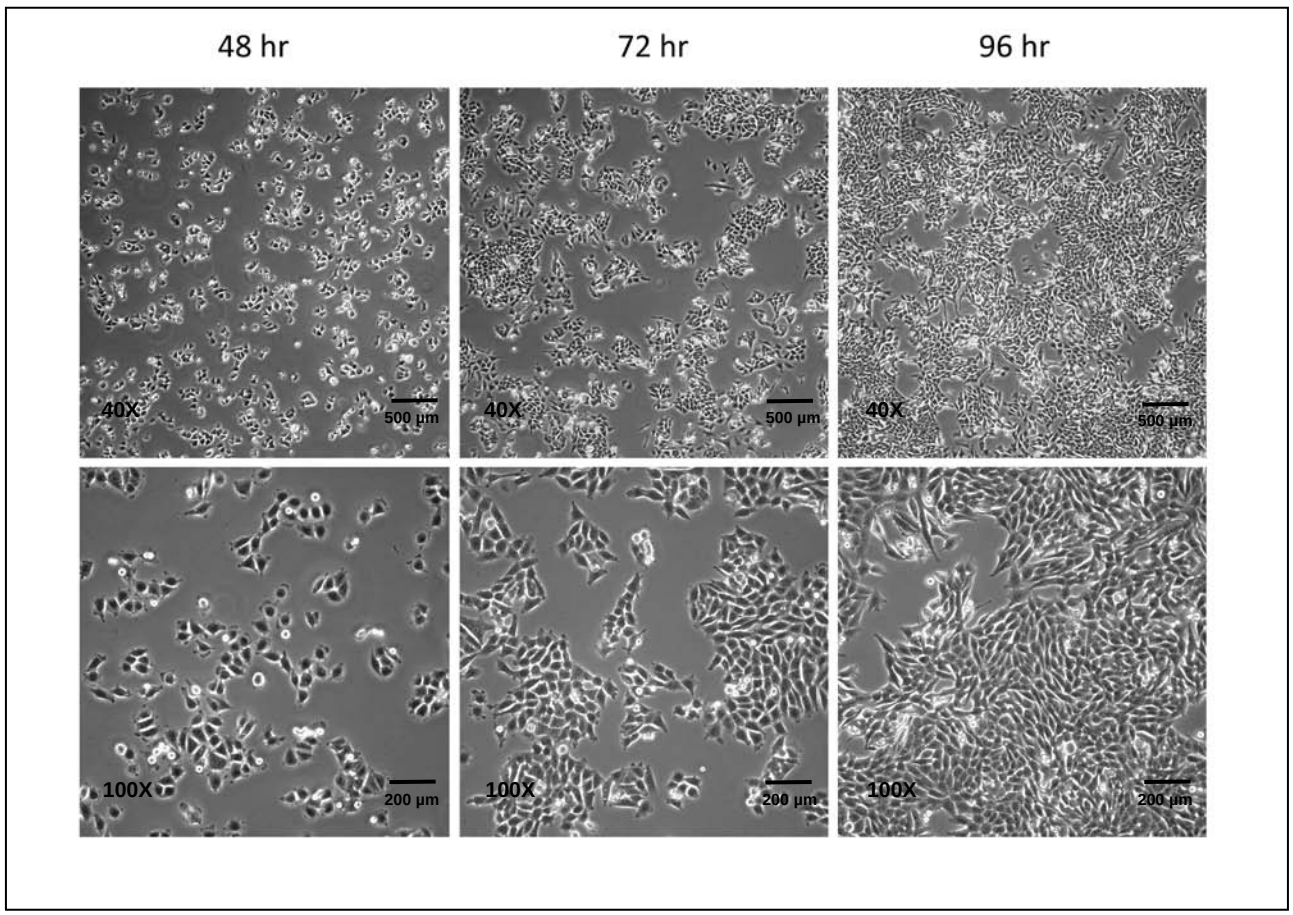


Figure 3: Photomicrograph of A-375 cells at various time points after seeding at a cell density of 2.5 x 10^4 viable cells/cm^2.

APPENDIX 2: GROWTH PROFILE OF NCI-PBCF-CRL1619 (A-375) CELLS

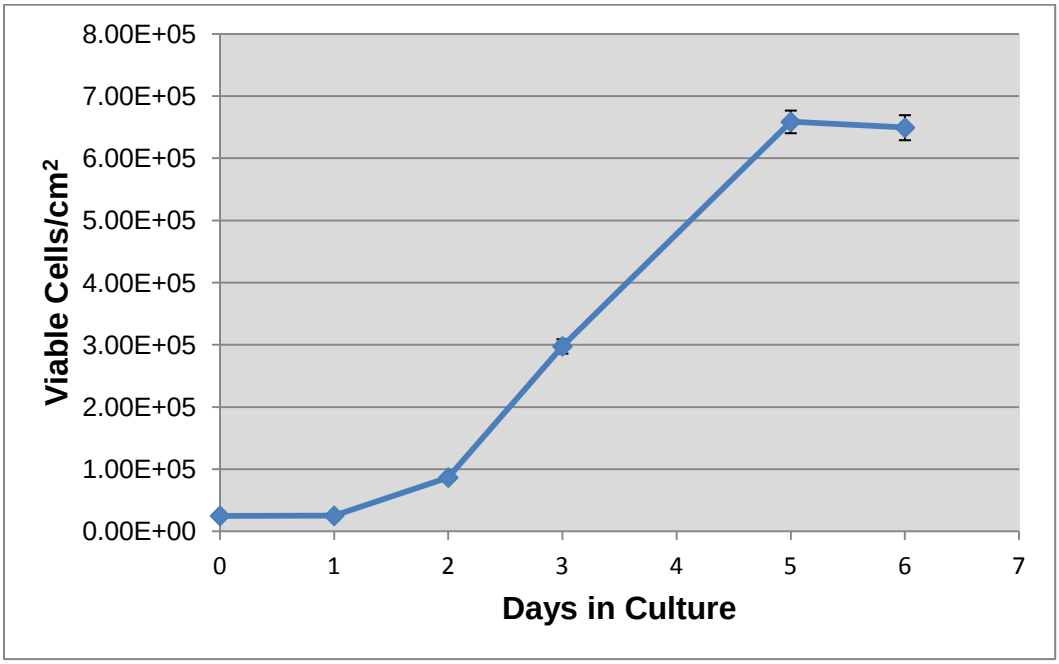
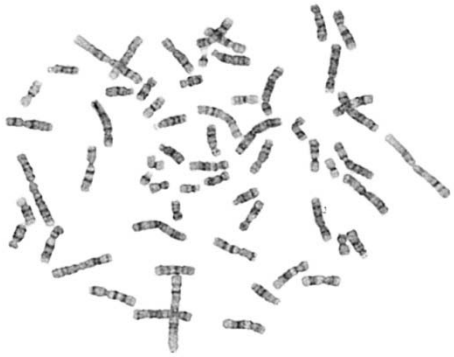
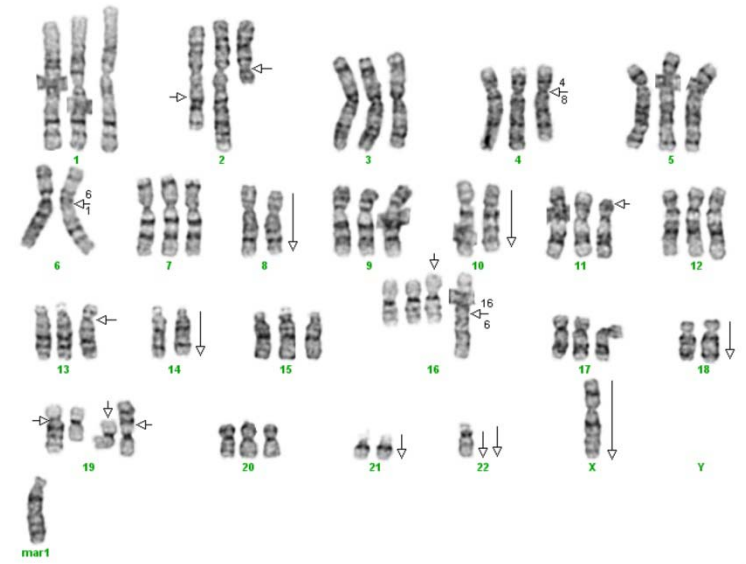


Figure 4: Growth curve for A-375 cells; cells were plated at 2.5 x 10^4 viable cells/cm^2; population doubling time (PDT) is approximately 32 h.

APPENDIX 3: CYTOGENETIC ANALYSIS OF NCI-PBCF-CRL1619 CELLS

NCI-PBCF-CRL1619 Karyotype Results	
 **Metaphase Spread**	 **Karyotype**

Number of metaphase spreads counted	10
Band level	300-400
Number of metaphase spreads karyotyped	10
Chromosome range	60-63
Sex	Female
Comments	Aneuploid*

Karyotype:

60-63,XX,-X,add(2)(q10),add(2)(q21),der(4;8)(p10;q10),der(6)t(1;6)(q12;q13),-6.-8,-10, del(11)(p15.1),add(13)(p10),-14,der(16)t(6;16)(q13;q12.1),+16,-18,add(19)(p13), add(19)(q10),+19,-21,-22,-22,+mar1[cp10]

(ISCN nomenclature written based on a triploid karyotype).

*Human diploid karyotype (2N): 46,XX (female) or 46,XY (male)

Karyotype Summary:

In the karyotype image, arrows indicate regions of abnormality. It should be noted that the karyotype description includes the observed abnormalities from all examined metaphase spreads, but due to heterogeneity, not all of the karyotyped cells will contain every abnormality.

This is an aneuploid human cell line of female origin containing 60 to 63 chromosomes per metaphase spread (hypotriploid). Structural abnormalities include rearrangements to chromosomes 1, 2, 4, 6, 8, 11, 13, 16, and 19, with most of the chromosome markers present in almost all of the examined cells.

The rearrangements include:

- Addition of unknown material to the short arms (designated by p) of chromosomes 13 and 19;
- Addition of unknown material to the long arms (designated by q) of chromosomes 2 (two different markers) and 19;
- Deletion of material from the short arm (designated by p) of chromosome 11;
- A translocation between chromosomes 4 and 8 involving the entire short arm of 4 and the entire long arm of 8 [der(4;8)(p10;q10)], a translocation between the long arms of chromosomes 1 and 6 [der(6)t(1;6)(q12;q13)] and a translocation between the long arms of chromosomes 6 and 16 [der(16)t(6;16)(q13;q12.1)];
- An unidentified marker chromosome was present in 3 cells [+mar1].

Numerical changes are based on a triploid karyotype which would contain three copies of each chromosome (3N). Therefore designations such as +16 indicates the presence of four copies of structurally normal chromosome 16 per metaphase spread and -6 indicates two copies of structurally normal chromosome 7 per metaphase spread.

(ISCN 2009: An International System for Human Cytogenetic Nomenclature (2009), Editors: Lisa G. Shaffer, Marilyn L. Slovak, Lynda J. Campbell)

Karyotype Procedure:

- **Cell Harvest:** Cells were allowed to grow to 80-90% confluence. Mitotic division was arrested by treating the cells with KaryoMax® colcemid for 20 minutes to 2 hours at 37°C. Cells were harvested using 0.05% Trypsin-EDTA, treated with 0.075M KCL hypotonic solution, and then fixed in three changes of a 3:1 ratio of methanol: glacial acetic acid.

- **Slide Preparation:** Slides were prepared by dropping the cell suspension onto wet glass slides and allowing them to dry under controlled conditions.

- **G-banding:** Slides were baked one hour at 90°C, trypsinized using 10X trypsin-EDTA, and then stained with Leishman's stain.

- **Microscopy:** Slides were scanned using a 10X objective and metaphase spreads were analyzed using a 100X plan apochromat objective on an Olympus BX-41 microscope. Imaging and karyotyping were performed using Cytovision® software.

- **Analysis:** Twenty metaphase cells were counted and analyzed, and representative metaphase cells were karyotyped depending on the complexity of the study.

Summary of Karyotyping Procedure:

G-band karyotyping analysis is performed using GTL banding technique: **G** bands produced with **t**rypsin and **L**eishman. Slides prepared with metaphase spreads are treated with trypsin and stained with Leishman's. This method produces a series of light and dark bands that allow for the positive identification of each chromosome.

A-375 cell line karyotyping was carried out by Cell Line Genetics, Inc. (Madison, WI 53719)

APPENDIX 4 : GLOSSARY OF TERMS

Confluent monolayer: adherent cell culture in which all cells are in contact with other cells all around their periphery and no available substrate is left uncovered.

Split ratio: the divisor of the dilution ration of a cell culture to subculture (e.g., one flask divided into four, or 100 mL up to 400 mL, would be split ratio of 1:4).

Subculture (or passage): the transfer or transplantation of cells, with or without dilution, from one culture vessel to another.

Passage No: the total number of times the cells in the culture have been subcultured or passaged (with each subculture the passage number increases by 1).

Population doubling level (PDL): the total number of population doublings of a cell line since its initiation in vitro (with each subculture the population doubling increases in relationship to the split ratio at which the cells are plated). See *Appendix 2* and *Appendix 7*.

Population doubling time (doubling time): the time interval, calculated during the logarithmic phase of growth in which cells double in number.

Seeding density: recommended number of cells per cm^2 of substrate when inoculating a new flask.

Epithelial-like: adherent cells of a polygonal shape with clear, sharp boundaries between them.

Fibroblast-like: adherent cells of a spindle or stellate shape.

APPENDIX 5 : REFERENCE

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2. Giard DJ, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51: 1417-1423, 1973. PubMed: 4357758
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APPENDIX 6: REAGENT LOT TRACEABILITY AND CELL EXPANSION TABLES

Table 4: Reagent Lot Traceability

Reagent	Vendor	Catalog #	Lot #	Expiration Date

APPENDIX 7: TABLE 5: CELL EXPANSION TABLE

Table 5: Cell Expansion Table

	FROM			FLUID CHANGE		Observation under microscope	CELL COUNT			TO		
By / Date	**Flask qty; size**	**Pass #**	**% Confluence**	**Add/ Replace**	**Volume (in mL)**		**Viable cells/mL**	**Total viable cells**	**% Viability**	**Split Ratio**	**Flask qty; size**	**Pass # PDL#**
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								

APPENDIX 8: CALCULATION OF POPULATION DOUBLING LEVEL (PDL)

Calculate the PDL of the current passage using the following equation:

$$PDL = X + 3.322 (\log Y – \log I)$$

Where: X = initial PDL
I = cell inoculum (number of cells plated in the flask)
Y = final cell yield (number of cells at the end of the growth period)

APPENDIX 9: SAFETY PRECAUTIONS

- Use at least approved Biological Safety Level 2 (BSL-2) facilities and procedures.
- Wear appropriate Personal Protective Equipment (PPE), such as isolation gown, lab coat with sleeve protectors, face shield and gloves.
- Use safety precautions when working with liquid nitrogen, nitrogen vapor and cryogenically cooled fixtures.
- Use liquid nitrogen freezers and liquid nitrogen tanks only in areas with adequate ventilation. Liquid nitrogen reduces the concentration of oxygen and can cause suffocation.
- Wear latex gloves over insulating gloves to prevent liquid nitrogen from soaking in and being held next to the skin.

  Note: Liquid nitrogen is extremely cold and will cause burns and frostbite. Metal inventory racks, tank components, and liquid nitrogen transfer hoses exposed to liquid nitrogen or nitrogen vapor quickly cool to cryogenic temperatures and can cause burns and frostbite.

- Wear a full face mask when thawing and retrieving vials from liquid nitrogen freezer.

  Note: Danger to the technician derives mainly from the possibility that liquid nitrogen can penetrate the cryovial during storage. On warming, rapid evaporation of the nitrogen within the confines of such cryovial can cause an aerosol or explosion of the cryovial and contents.